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Human Papillomavirus and carcinogenesis: Novel mechanisms of cell communication involving extracellular vesicles

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ABSTRACT

A small group of mucosal Human Papillomaviruses are the causative agents of cervical cancer and are also associated with other types of cancers. Certain cutaneous Human Papillomaviruses seem to have a role as co-factors in the UV-induced carcinogenesis of the skin. The main mechanism of the tumorigenesis induced by Human Papillomaviruses is linked to the transforming activity of the viral E6 and E7 oncoproteins. However, other mechanisms, such as the gene expression control by specific microRNAs expression and deregulation of immune inflammatory mediators, may be important in the process of transformation. In this context, the release of Extracellular Vesicles with a specific cargo (microRNAs involved in tumorigenesis, mRNAs of viral oncoproteins, cytokines, chemokines) appears to play a key role.

1. Introduction

The large group of Human Papillomaviruses (HPVs), that includes more than 220 genotypes, are double-stranded DNA viruses that infect mucosal and cutaneous epithelia. They are classified in five genera (α, β, γ, μ and ν) into the Papillomaviridae Family, including viruses infecting all vertebrates. The HPV genomes are about 8 kb in length, organised in 3 functional regions: a Long Control Region (LCR), also known as upstream regulatory region (URR), the Early and the Late regions. LCR contains cis elements for transcription and replication of the viruses; the early region encodes E6, E7, E1, E2, E4 and E5 regulatory proteins while the late region encodes the structural L1 and L2 proteins able to assemble in ecosaedric structures. Coding region resides in a unique DNA strand. E5 protein is lacking in β and γ genera while in γ-HPV101, 103, and 108 is lacking also E6. Several HPVs encodes an alternatively spliced protein known as E8 ^ E2. This is a DNA-binding protein that competes with E2 acting as a transcriptional repressor, and represses E1/E2-dependent replication of the viral origin [1,2].

At least 12 HPVs of 4 species in the α genus, α9-HPV16, 31, 33, 35, 52, and 58, α7-HPV18, 39, 45, and 59, α5-HPV51 and α6-HPV56 are associated with neoplasia in the anogenital and upper respiratory human tracts and are defined high-risk (HR) genotypes by epidemiological studies. In anogenital and oropharyngeal cancers the HR-HPV genomes are integrated in the host chromosome, fact considered high priority in the induction of carcinogenesis. The most oncogenic α-type is HPV16, the causative agent of more than 60 % of all cervical cancers, and also the prevalent type in other anogenital and head and neck cancers. The E6 and E7 oncoproteins of the high risk genotypes are tumor-specific and tumor rejection antigens, expressed in tumors and precursor lesions, ideal targets for immunotherapy [3]. E6 and E7 contribute to viral immunoevasion and act in concert to promote tumor development through the interaction with multiple cellular proteins. E6 binds to the p53 tumor suppressor through the ubiquitin ligase E6-AP and to pro-apoptotic Bcl2 members proteins, and inhibits pro-caspase-8 activation to prevent cell apoptosis. E7 mainly affects factors involved in cell proliferation and cell cycle regulation, such as pRb, cyclins and cyclin-dependent kinase inhibitors. The maintenance of the malignant phenotype, the genomic instability and the transformation of primary human keratinocytes are mainly due to the continuous expression of E6 and E7 proteins of HR-HPVs.

The beta HPVs are subdivided into five species (beta1-5), have cutaneous tropism and together with HPVs of the γ genus, are abundantly present on the skin of healthy individuals as part of the normal skin

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microbiota [4]. The fact that impairment of the immune system, as in organ transplant recipients (OTRs), is highly associated with the risk of Squamous Cell Carcinoma (SCC) development, strongly supports the involvement of these viruses in skin cancer. The first β-HPVs, HPV5 and 8, were isolated from patients suffering from the genetic disorder epidermodysplasia verruciformis (EV) [5]. These individuals have a high susceptibility to β-HPV infection as well as to the SCC development in solar-exposed skin regions. A recent meta-analysis showed that five β-HPV types, i.e., HPV5, 8, 17, 20, and 38, are significantly associated with risk of SCC [5]. β-HPV types may represent a novel group of oncogenic HPVs in addition to α-HPVs. However, α and β-HPVs act with a different mechanism in promoting carcinogenesis. While the former group is required throughout the entire carcinogenic process, the latter appears to play a role only at an early stage of skin carcinogenesis, by facilitating the accumulation of DNA damage induced by UV radiation. E6 and E7 oncoproteins from some β-HPV types, e.g., 5, 8, 23, 38 and 49, display transforming activities, being able to deregulate key pathways involved in cell proliferation. Experiments in animal models have further corroborated the *in vitro* data and provided clear evidence for the cooperation of the viral proteins with UV radiation in promoting SCC. Importantly, the *in vivo* models consisting in transgenic mice harbouring E6 and E7 of beta-types confirmed the hypothesis of the "hit-and-run" mechanism of HPV in the UV-mediated skin carcinogenesis [6–10]. The beta1 types, HPV5 and HPV8, are classified as 'possibly carcinogenic' in the IARC classification (International Agency for Research on Cancer) of carcinogenetic substances. Recent studies point to a cross-talk of beta-HPVs with the cell-autonomous immunity of the host keratinocytes and the local immune microenvironment that determines the fate of cutaneous HPV infection and the penetrance of disease [9].

It has been reported that HPV^+ cells release Extracellular Vesicles (EV) thus modifying the microenvironment, affecting tumor development and chemoresistance [11–14].

EVs are generically defined as vesicles released by virtually all cell type into interstitial spaces as well as in every body fluids from blood to urine and saliva (for review see [15,16]). They include exosomes (Exos), microvesicles (MVs), and apoptotic bodies (ABs) and have recently attracted great attention in cancer research. Their lipid bilayer membrane envelops all type of biologic macromolecules ranging from miRNAs, mRNAs, proteins, free metabolites. Of course, membrane lipids themselves could be also considered as part of EVs cargo able to be transferred to recipient cells [17]. EVs are important players in intercellular communication during normal homeostatic regulations, then it was not surprising to reveal the ability of cancer cells to hijack this way of signaling to induce the transformation of non-malignant cells as well as to subvert the tumor microenvironment (TME), making normal cells able to improve tumor growth [18,19]. In addition, it has been reported that cancer patients display an increased number of circulating EVs compared to healthy subject, suggesting that EVs not only are part of the TME subverting program, but also have a role in metastasis through the generation of the so called pre-metastatic niches. The idea of pre-metastatic niches directly descends from the "seed and soil" theory formulated by Steven Paget and provides a possible explanation of why certain tumors are able to metastasize only specific tissues. According to this hypothesis, the ensemble of factors secreted by tumor primes the secondary site to receive metastatic cells and EVs are important mediators of pre-metastatic niches generation, acting via several mechanisms [20]. Indeed, due to the expression of a specific pattern of integrin, different populations of EVs could be addressed to different tissue thereby priming them to metastasis implant [21]. Further, the idea of a unidirectional flow of "information" from tumor to neighboring and distant normal cells has been recently challenged and several lines of evidence suggest that EVs secreted by neighboring normal cells are also able to influence tumor growth. This is, for example, the case of Cancer Associated Fibroblast (CAFs)-derived EVs that enhance the growth of PC3 prostate cancer cell line by increasing glucose metabolism and downregulating their mitochondrial function [22].

2. Mucosal HPVs and extracellular vesicles

Whereas several reports addressed the question of how HR-HPVs are able to interfere with the cyto-chemokine network to subvert microenvironment, thereby escaping innate and acquired immune responses (for reviews on this aspect see [23]), only few studies addressed the question of how HPVs oncogenes expression alter the EVs cargo. Seminal observation aimed to characterize specific modification of EVs was addressed for the first time in 2013 by Honegger et al. [11], even if some clues of the presence of EVs in supernatant collected from HPV^+ cell lines could be found already in 2009 and later on in 2011 [24,25]. Honegger *et al.* reported that EVs isolated from $HPV18^+$ HeLa cells overexpressed Survivin, an antiapoptotic protein associated to tumor progression and chemoresistance [26]. Survivin was reported to be regulated in cells by E6 through p53 downmodulation [27] and it was specifically directed to vesicular pathway, whereas other members of IAP family to whom Survivin belongs (*i.e.* XIAP, Livins, and c-IAP1) were not targeted to EVs. This effect is also dependent on E6/E7 expression as oncogenes ablation by siRNA decrease Survivin loading into EVs. Another striking feature reported in this study, was the lacking of both E6 and E7 into EVs that the authors recognized as mainly consisting of exosomes, due to the expression of exosomal markers as Hsp70, CD9, CD63, Tsg101, β-actin and annexin-1. They also reported that the silencing of E6 and E7 reduced the amount of EVs release even if this effect was measured only by indirect methods (i.e. Acetyl-Cholinesterase activity in the supernatants of HeLa cultures and total protein measurement) [11].

The same group reported two years later a deep miRNAs analysis from both HeLa cells and EVs isolated thereof [12]. They observed that, compared to parental cells, the exosome enriched fraction is also enriched in small RNAs in the range of 20–40 nucleotides, compatible in length with miRNAs. Using small RNA deep sequencing analysis, they identified 47 miRNAs abundantly expressed in EVs from both control or E6/E7-silenced HeLa cells. Between these, 21 were upregulated and 4 were downmodulated more than 1.5 fold after E6/E7 silencing.

It has been also demonstrated that, at least in the case of HeLa cells, exosomes thereof derived not only carried specific proteins as Survivin and miRNAs but they are also able to convey long non-coding RNAs (*i.e.* lncRNAs) as lincRNA-p21, CCNDA1-ncRNA, HOTAIR, TUG1 and GAS5 [28]. Also in that case, authors performed exosomes isolation by sequential centrifugation and they checked the expression of CD63 exosome-specific marker. Between the lncRNAs tested, lincRNA-p21 is an interesting regulatory RNA as it represses p53-dependent responses [29], thereby implying a possible role of lincRNA-p21 in the gene expression of EVs acceptor cells.

To date only two studies described EVs and their cargo from HPV^+ genital clinical specimen [30,31]. Liu et al., reported that in cervicovaginal lavages derived from healthy (HPV^-) subject as well as from HPV^+ and cervical cancer patients, the levels of both miR-21 and miR-146a were directly correlated with the progression to cancer, being minimal in healthy subject and maximal in squamous cell carcinoma patients. Also in this case, the procedure used to isolate EVs allowed the enrichment of the exosomal fraction as indicated from the expression of CD9 and CD63 markers [30]. These authors also demonstrated the dependence of EVs release from intracellular calcium rise as the treatment of HeLa cells with the calcium ionophore A23187 increased the number of secreted EVs and, by consequence, the amount of miR-21 and miR-146a detected in the exosomal but not in the cellular fraction. Another striking feature reported, was the ability of miR-21 to inhibit a miR-21-sensitive reporter gene in recipient cells incubated with EVs derived from HeLa cells, thereby demonstrating that EVs-associated miR-21 is functional in acceptor cells. Two years later, another Chinese group demonstrated by q-RT-PCR, again from cervicovaginal lavages specimens, the presence of HOTAIR, MALAT1, and MEG3 lncRNAs and

that the expression of these lncRNAs were significantly different in HPV$^+$ patients compared to healthy individuals, being even higher in cervical cancer patients [31].

We and others have characterized the EVs production in HPV16 E6 and E7 retrovirally transduced primary human keratinocytes [13,32]. Using nanoparticle track analysis on preparations of chemically precipitated exosomes/EVs Harden et al., reported three main classes of EVs of 67, 89 and 121 nm diameter. These EVs were screened for the expression of a panel of 68 cancer-associated miRNAs and their expression was compared to that recorded in producing cells. These authors found 16 miRNAs similarly regulated in both E6/E7 transduced keratinocytes and EVs. Most of them were upregulated whereas only few were downregulated. Further, seven miRNAs were differentially expressed in EVs *versus* producing cells. A metanalysis of these deregulated miRNAs revealed that they are involved in the regulation of several pathways related to cellular transformation as cell growth, proliferation and cell death and survival [32]. On the other hand, our group reported that EVs isolated from HPV-16 E6/E7 expressing keratinocytes as well as from HPV16$^+$ squamous cell carcinoma SiHa cell line contain both E6 and E7 mRNAs and are able to reduce the expression of p53 in acceptor keratinocytes [13]. Recently, we also provided evidence that EVs isolated from keratinocytes expressing E6/E7 from HPV16 have a peculiar pattern of inflammatory cyto-chemokine mRNAs (Fig. 1), which somehow differs from those in parental cells [14]. Our results on cyto-chemokines mRNAs are reminiscent of those obtained by Rana and co-workers that reported the ability of Poly(I:C) to increase the amount of EVs-associated IL-36γ in HPV$^-$ keratinocytes [33]. As HPV is able to inhibit Poly(I:C)-dependent induction of pro-inflammatory genes [34], it is plausible to hypothesize that, as we reported for many pro-inflammatory genes, also IL-36γ is down-modulated by HPV.

Even if most of the studies on cancer-associated EVs are mainly focused on the proprieties of exosomes and microvesicles, at least in the case of HR-HPV, it has been reported that also apoptotic bodies (ABs) are able to transfer their tumor-associated cargo to acceptor cells. Indeed, ABs collected from HPV-16 and HPV-18 positive cell lines transfer HPV DNA to human primary fibroblast and induce anchorage-independent growth ability, an hallmark of transformation [35], in the latter cells. Collectively, these results suggest the ability of these large EVs to transform recipient cell [36]. The same group also reported that late ABs are taken up by fibroblasts more efficiently than early ABs, nevertheless the phagocytic activity of these cells remain low compared to those exerted by professional phagocytic cells [37].

Besides the development of anogenital squamous cell carcinoma,

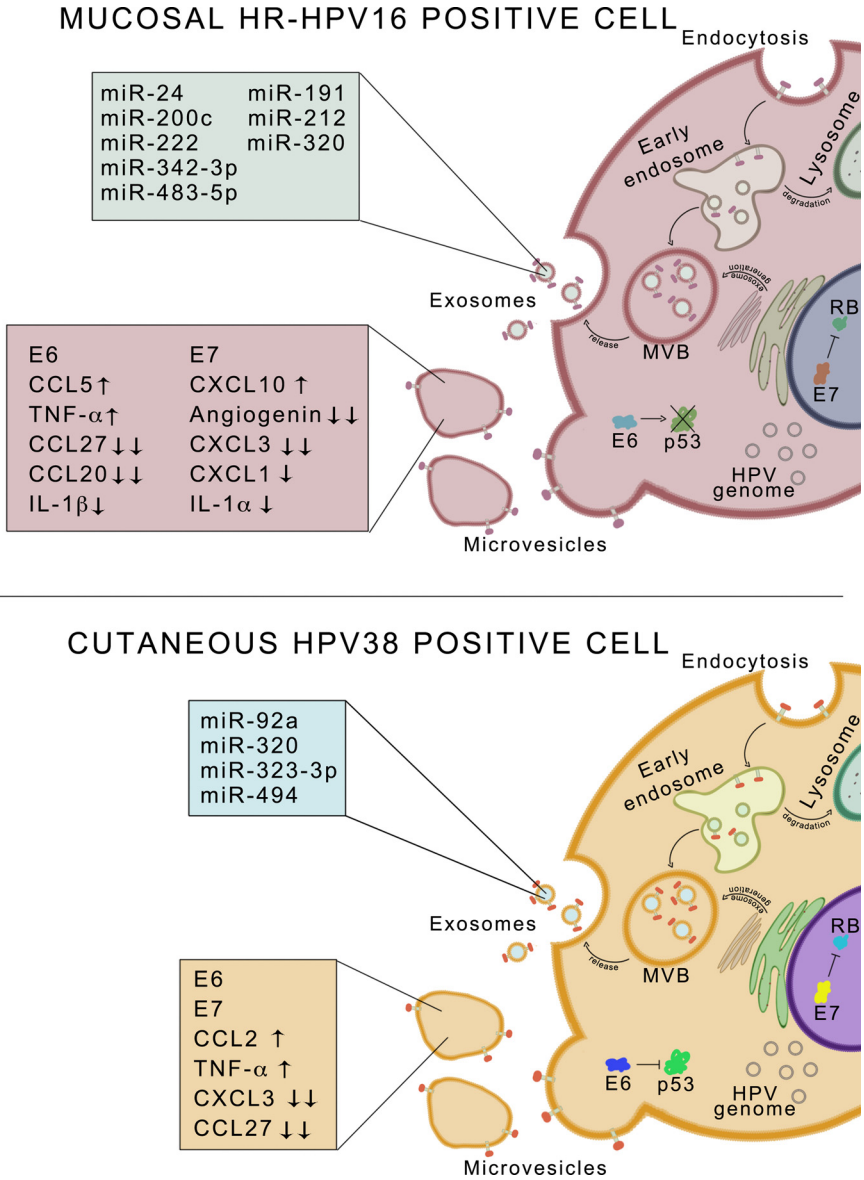

Fig. 1. Biogenesis, secretion and cargo content of Extracellular Vesicles from HPV positive cells. EVs generated by outward budding and shedding from the plasma membrane (microvesicles or shed-microvesicles) or formed within multivesicular bodies (MVBs) as intraluminal vesicles (ILVs) released upon fusion of MVBs with the plasma membrane (exosomes), contain a specific set of bioactive molecules depending on both their biogenesis and HPV genotype (mucosal, upper panel or cutaneous, lower panel). In the boxes are reported specific microRNAs and mRNAs of viral oncoproteins as well as of inflammatory cytokines and chemokines delivered by EVs from mucosal HPV16 or cutaneous HPV38 E6 and E7 transduced cells. Arrows indicate the up- or down-regulation of mRNA expression with respect to EVs from control keratinocytes.

HR-HPVs genotypes were also associated to a different degree to oropharyngeal cancer, especially in the tonsillar region and at the base of tongue [38,39]. Despite a stable incidence rate of total oropharyngeal squamous cell carcinomas, HPV^- tumors decrease whereas HPV^+ increase rapidly becoming a real health emergency [40]. Even if HPV infection was associated with a percentage of total oropharyngeal cancer estimated between 10 and 50 % [41], it often has a poor clinical outcome due to a high rate of recurrence in the same anatomical district or in unusual sites. In addition, the recurrence of HPV^+ oropharyngeal cancer manifests later than in HPV^- patients [38,42–44]. Using whole genome sequencing analysis of HPV^+ oropharyngeal cancer specimen, it has been identified a panel of mutated genes involved in malignant progression including several members of Mucin family, HLA-A, -B and DRB1 as well as the surface marker CD172a/SIRPA [45]. These mutations are responsible for the overexpression of MUC16 and SIRPA in HPV^+ oropharyngeal cancer as well as in circulating exosomes isolated from blood of patients. EVs derived from HPV^+ oropharyngeal cancer specimen possess the ability to induce both epithelial to mesenchymal transition (EMT) and increase the migration and invasion of a HPV^- mammary epithelial cell line and, contrary to EVs derived from anogenital squamous cell carcinoma, they express HPV16 E7 [45].

In another study, EVs isolated from both HPV^+ and HPV^- oropharyngeal squamous cell carcinoma (OPSCC) cell lines were compared [46]. Using Tunable Resitive Pulse Sensing measurements, these authors reported that HPV^- OPSCC cells produced more EVs than HPV^+ cells. In addition, at least one of the HPV^- OPSCC cell line released EVs with a larger diameter compared to HPV^+ cells. Also in this case, small RNA sequencing was performed to identify miRNAs specifically associated with EVs from both HPV^+ and HPV^- OPSCC cells. This analysis revealed that 14 miRNAs were enriched in EVs from HPV^+ cells as miR-9, -20b, and let-7b. On the other hand, 19 miRNAs were overrepresented in EVs from HPV^- cells, between these miR-29. Nine miRNAs including miR-20a, -23, -26 and -27 were highly expressed in all EV independently of HPV status. A metanalysis of the predicted, cancer-associated, cellular pathways targeted by these miRNAs indicated that some overlap exists between HPV^+ and HPV^- EVs-associated miRNAs targets. In particular, PI3K-Akt, FoxO, HIF-1, mTOR and p53 signalling pathways are affected by both, even if HPV^+ EVs-associated miRNAs seemed to influence a greater number of cellular target involved in these pathways.

3. Cutaneous HPVs and extracellular vesicles

More than 50 β-HPVs have been classified so far, albeit other β-types exist, since partial genome sequences of novel putative β-HPV types have been shown [47]. There are five different species of beta HPV types: β1, β2, β3, β4, and β5. Beta1 and β2 HPVs are the most common types in the skin, while the other species include very few HPV types detected also in sites different from skin: β3 (n = 4, HPV49, 75, 76 and 115), β4 (n = 1, HPV92), and β5 (n = 2, HPV96 and 150) [48]. Beta HPV DNA is frequently present in the skin of immunocompetent individuals, with prevalence estimates ranging from 39 % to 91 % (18–20). The presence of DNA of β-HPVs in the skin of infants and young children indicates that this infection is acquired early in life, probably through direct contact with the skin of the parents [49]. Serological studies measuring antibodies against type-specific L1 protein of β-HPV provide evidence that the exposure to cutaneous HPVs is common [50].

Beta HPVs have intrinsic properties to promote inflammatory responses in epidermodysplasia verruciformis patients [51], suggesting that this can lead to chronic inflammation and favor tumor progression. The cross-talk between β-HPVs and the immune system is exerted at various stages of infection. The interactions of β-HPV proteins with immune signaling pathways in the host cell enable the virus to persist by the evasion of the immune control.

In *in vitro* studies, toll-like receptor 9 expression is induced by UV-mediated signals and other stresses, but β-HPV38 E6 and E7 proteins have the ability to repress toll-like receptor 9 expression, like mucosal HR-HPV [52–54].

Beta-HPVs, when sufficiently expressed, can promote the initial steps of skin carcinogenesis promoted by UV. They carry out this process by expanding the UV-sensitive stem/progenitor cell compartment, prolonging local UV-induced immunosuppression by preventing the repopulation of the epidermis with Langerhans cells, promoting the lifespan of their host cells through prevention of UV-induced apoptosis. Once critical genetic alterations are established, such as mutations in the tumor suppressor p53, β-HPVs may become dispensable for the maintenance of the malignant phenotype. However, disease penetrance is controlled by host restriction factors and extrinsic immunity. Once these 'brakes' are released, viral expression and replication can occur, with all their deleterious consequences in the general population.

In the recent years, the role of extracellular vesicles (EVs) has been studied in carcinogenesis and it has been shown that their production and release is deregulated in cancer [55–57]. The possible eff ;ects on the intercellular communication of HPV^+ cancer cells is not yet well known. The release of EVs from cancer cells can impair the microenvironment, aff ;ecting tumor development and chemoresistance [58,59]. Moreover, rising evidence suggests that cancer cells use EVs transmitted nucleic acids and proteins to evade an immune response [60].

The role of HPV E6 and E7 proteins of HPV38 in the modulation of the inflammatory microenvironment has been investigated in our studies. The expression of the HPV proteins in human keratinocytes leads to the down-modulation of a series of inflammatory cyto- and chemokines and affects the inflammatory immune mediators delivery through the EVs. Indeed, we have shown the increase of the expression of CCL2 and TNFα in EVs derived from $HPV38^+$ keratinocytes, whereas CXCL3 and CCL27 expression was downregulated (Fig. 1). It is conceivable that the modulation of the EV cargo by HPV E6 and E7 could have a role in the alteration of the microenvironment as well as in the regulation of cellular functions in non-infected recipient cells and/or immune surrounding cells, through the transfer of EV content [14].

We have shown that cutaneous HPV38 E6 and E7 expression is able to modulate microRNAs carried by EVs (Fig. 1), in particular microRNAs involved in tumorigenesis [13]. It has been reported that E6 and E7 oncoproteins from high-risk $HPV18^+$ squamous carcinoma HeLa cells are able to modulate the number and the contents of exosomes. Moreover, the microRNA cargo of exosomes released by HeLa cells is dependent on the expression of E6 and E7 oncoproteins [11,12]. Interestingly, Epstein-Barr Virus encoded miRNAs are delivered via exosomes and affect known targets in recipient cells. Hepatocellular carcinoma cells secrete exosomes with increased content of specific miRNAs that can epigenetically modulate gene expression and induce cellular transformation [61].

It is likely that the delivery of microRNAs by HPV-infected cells to the non-infected recipient cells is able to induce tumorigenesis through the effect of these microRNAs on their targets.

In addition, we have shown that EVs produced by HPV^+ keratinocytes have the capability to deliver viral oncogenes [13]. Therefore, HPV E6 and E7 proteins can affect the extracellular milieu and potentiate the virus-induced tumorigenesis through EV delivery.

4. Conclusions and future studies perspectives

As opportunistic parasites, viruses possess the intrinsic ability to hijack intracellular signalling pathways and cellular microenvironment, taking advantage to complete their own replicative cycle. In the case of tumorigenic viruses, as HPVs, this attitude is exploited to promote both cancer growth and tumor dissemination through a deeply reprogramming of cell released cyto-/chemokines and EVs. As soluble factors and EVs represent the two sides of the same coin, nowadays it has become common the use of the term "secretoma" to indicate the whole set of

entities released by a cell. The reported evidence underlines how HPVs hijack the cyto-/chemokine network as well as the EVs route of intercellular transmission, thereby altering the cell secretoma. Nevertheless, we are just at the beginning of an era in which EVs can be used for prognostic and diagnostic purposes and some fundamental questions are opening.

Most of the studies have been performed on HR-HPVs belonging to α genus, but it has been recently reported that members of the β genus (*i.e.* HPV49) have functional similarities with HPV16 at least in transgenic mice [62]. Are these β-HPVs able to modify the infected cells secretoma in the same way? Do quantitative and/or qualitative differences exist between EVs released from cells expressing mucosal versus cutaneous HPVs in term of cargo content? Do the EVs released by different type of cancer-associated HPVs have same migratory properties and, then, the ability to form similar pre-metastatic niches or difference exists? As in the case of other oncoviruses, especially those having a DNA genome, some reports indicate the putative presence of different genotypes of viral miRNAs in the HPV genome [63]. Four out of five HPV (*i.e.* two from HPV16, one from HPV38 and one from HPV68) miRNAs were validated and their expression was verified in established cell lines as well as in clinical specimens even if at low expression level [64]. Interestingly enough, the analysis of putative targets of two HPV16-codified miRNAs revealed that many target genes involved in cell cycle, immune responses and cell migration are commonly regulated by both miRNAs that have also two target sites in the viral genome (*i.e.*, LCR region and L1 gene respectively) [63]. Even if the low cellular expression of these viral-derived miRNAs makes them not attractable as diagnostic biomarkers, are they uploaded and enriched into EVs? Could the spreading of these viral miRNAs play a role in the onset of metastasis or in the recurrence of disease? Could EVs and their content be exploited as prognostic/diagnostic markers to define HPV-associated cancer progression or transition between low grade, non-pathogenic lesions, to pre-cancerous and cancerous ones?

All these unanswered questions deserve more studies to improve our knowledge on HPVs biology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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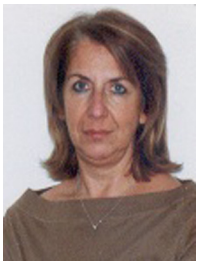

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